Chapter 7

Summary, Conclusions and Perspectives

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The mechanosensitive channel of large conductance (MscL) is until today one of the best-studied mechanotransduction channels. Generally, mechanosensitive channels are triggered by physical perturbation of the membrane and respond to this stimulus by opening an ion conduction pathway. By that, they are one of the components in charge of controlling the passage of ions and small solutes across the membrane. This conversion of mechanical triggers (membrane stretch) into chemical signals (ion flux) plays an important role in signaling pathways, but also in other processes like e.g. osmoregulation (24). Our 'simple model system' MscL serves as *E. coli's* last resort emergency release valve upon hypoosmotic shock (23.). Bacteria face this shock from high salt to low salt for instance when it starts raining after a longer dry period. By firing solutes through a large (30-40 Å) (32, 136) nonselective pore, opening at critical tension in the membrane, MscL prevents rupture and maintains the cellular integrity (24).

However, although MscL comprises a simple architecture of five identical subunits (27), its activation depends on membrane tension only (127). Even though a large body of experimental data is available, there remain two crucial questions to be answered: How membrane stress is sensed before it is translated into large structural rearrangements, and how does the channel physically open the conducting pathway (137)?

This thesis aims at solving one of the major challenges to address these questions: the homooligomeric nature of MscL. Since a single gene encodes MscL, mutations are by definition always present in five-fold symmetry of the subunits. Thereby, changes of a single residue within the pentamer are very challenging. As a result, all studies so far, deal with averaged information of all subunits, whether it is a mutation (21, 93, 101) or the position of a reporter molecule like e.g. a spin label in electron paramagnetic resonance (EPR) spectroscopy (104). Based on this average information from experimental data, and for simplicity, many of the empiric models assume a symmetric, coordinated rearrangement of the subunits during the course of gating.

Stop being average! *In vivo* heterooligomerization of MscL

To overcome these limitations, thus eliminate averaging information of the homopentamer, we developed a toolbox comprising *in vivo* heterooligomerization and biochemical isolation/characterization steps to obtain single-subunit resolution (**Chapter 2**). By extending the set of MscL building blocks (subunits) from a single to two gene products (WT-*Strep*II and G22C-His MscL), realized by duet-gene-expression of different *mscL* variants in *E. coli*, we obtained heteropentamers. This diversification of the subunit composition, achieved while leaving the MscL biogenesis to *E. coli's* natural resources, overcomes some of the restraints of previous attempts, like e.g. tandem gene expression, where several MscL copies were genetically fused together (42).

However, the sample present in the bacterial membrane is still a mixture of all possible permutations. To reach our goal of obtaining single subunit resolution for MscL by means of a defined oligomer assembly, we need to sort the complexes based on their subunit composition. Since the building blocks are only slightly different, this procedure requests a cascade of

biochemical separation techniques. The two MscL subunit variants that we used in our studies are marked with different affinity tags. These tags, a His6- (HHHHHH) and a *Strep*II-tag (WSHPQFEK) allow separation by sequential two-step affinity chromatography. Fortunately, MscL remains a stable complex during detergent solubilization and biochemical isolation methods. Therefore, only heteropentameric MscL is able to bind to both affinity columns. Additionally, to their function in affinity chromatography, the tags also influence the electrochemical properties of MscL, i.e. the isoelectric point (pI). Depending on the ratio of the differently tagged subunits within pentameric MscL the p*I* changes and enables separation by chromatofocusing (121, 122). Thereby, heteropentamers of MscL can be resolved based on the subunit composition as WT_4G22C_1, WT_3G22C_2, WT_2G22C_3, and WT_1G22C_4 and the limitations arising from the homopentameric nature of MscL are eliminated.

A single hydrophilic residue unravels symmetry and gating mechanism

Gaining the control over the individual building blocks of functional MscL enables insights on how individual subunits contribute to the functional properties of MscL (**Chapter 4**). After almost 20 years of research, with mutant libraries (20, 21, 93, 145), crystal structures of two MscL homologues (Tb-MscL (27) and Sa-MscL (85)), EPR spectroscopy of the closed, a closed expanded and the open state (104), and MD simulations (28, 48, 86), the gating mechanism of MscL, or more precisely, the very first steps of gating remains enigmatic.

Thus, although we know the location of the gate (27, 123) and the residues critical for gating (3, 21, 60, 145) we cannot sketch a detailed map of the mechanism underlying gating. The functional description of the gating process, however, has shown large-scale structural rearrangements

opening up a pore as wide as 30-40 Å (32, 104). Furthermore, electrophysiology has shown defined subconducting states (substates) between closed and fully open state (3). These substates very likely present defined intermediate structural arrangements of MscL. But, what defines the threshold between closed and the first subconducting state? Molecular dynamics simulations of the last ten years have addressed this question, not only for MscL, but also for other channels with a comparable gate region (5, 11, 29). These channels share a motif known as hydrophobic girdle or, more common, hydrophobic gate (reviewed in **Chapter 3**). As the name indicates already, hydrophobic residues constrict a region that functions as the gate, notably without sterically occluding it. MD simulations of synthetic systems like carbon nanotubes (57) or *in silico* ion channel mimics (8) and recently voltage-gated synthetic ion channels (108) have shown that subtle changes in the hydrophobicity, i.e. making the pore surface more hydrophilic, perturbs the gate and allows for the flow of ions. Ion channels could also utilize this mechanism, by placing hydrophilic residues in the normally hydrophobic lining of the gate, by for instance helix rotation. A mechanism like this has been observed for the gating of the mechanosensitive channel of small conductance, MscS. In the closed state Leu-105 and Leu-109 form the hydrophobic gate (5), while during gating Gly-104 and Gly-108, the direct neighbors of the gate residues play an important role (38). In Eco-MscL Leu-19 and Val-23 form the gate. Like in MscS, their direct neighbors are hydrophilic residues (Asp-18 and Gly-22). Thus, also here a rotation of the helical backbone would achieve a change of the hydrophobicity of the pore lining. In **Chapter 4** we have addressed this hydrophobic gating hypothesis experimentally. With the heteropentamers of MscL as tool we were able to show that a single hydrophilic substitution in the gate region (G22C subunit with the

positively charged MTSET label attached) was able to disrupt the gate and opened the conducting pathway (**Figure 1**).

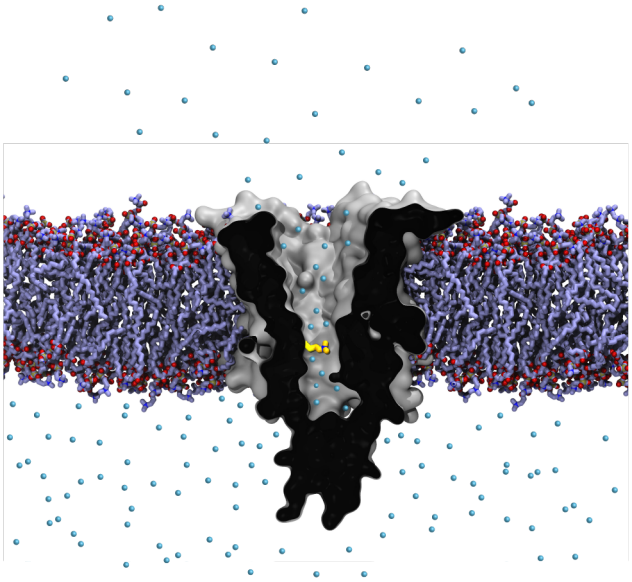

Figure 1 | Model of a cross section through Tb-MscL with a single MTSET moiety present in the gate region. A single charge or hydrophilic residue within the hydrophobic gate region is able to perturb the gate, allows hydration and thereby enables ion conductance. Image credits H.I. Ingólfsson, printed and modified with permission.

This activation, triggered by a single subunit, further proved that gating is an asymmetric process and backbone rotation of a single pore lining helix is enough to perturb the gate. Furthermore, the early substates that MscL visits during MTSET induced gating remained comparable to the ones observed for WT MscL activated by tension. Thus, *in situ* activated heteropentameric MscL has WT-like gating parameters. By that, hydrophobic gating of ion channels, a hypothesis that had lived for a decade in literature and got mainly support from computational studies, was evidenced at the experimental stage as the gating mechanism for MscL.

But, as often in science, answering one question raises a few new ones. In patch clamp experiments we mainly observed substates upon charge activation of MscL. This behavior was independent if one or five charges were placed in the pore by means of MTSET attached to G22C subunits, albeit it was more pronounced for the heteropentamers. The full opening, however, could only be observed upon additional application of tension. So, hydrophobic gating is not the only energetic barrier for gating. However, the fact that the mutant WT_4G22C_1 had a preference to populate early substates, thus can be considered locked in an early subconducting structural conformation, allowed us to define the geometrical dimensions of this state by molecular sieving experiments (**Chapter 5**). We supplemented this data with modeling of our electrophysiological data. Both analyses estimate the dimensions of the subconducting pore of WT_4G22C_1 to be up to 10 Å wide, while homopentameric $G22C_5$ opened to about 25 Å. Nevertheless, as we observed in patch clamp even MTSET triggered $G22C_5$ does not represent a fully open state. The additional energy, i.e. tension, required to fully open the channel remains substantial. Our experimental data is in good agreement with published data, where 60-70 % of the energy were required for preexpanding the channel (2, 128) and above that hydration of the pore occurred. The further conversion from subconducting to fully open state is less dependent on tension. Here, we reverse the order of activation and first hydrate the gate by means of increasing the hydrophilicity in the pore lining in absence of structural expansions as they can be evoked by tension in the membrane. When we subsequently apply tension to the membrane, MscL opens fully. Overall the hydrophobic gating indeed is one of the molecular mechanisms of gating in MscL, but the large structural rearrangements demand further energetic input (2).

The carboxyl-terminus of MscL - Highest level of sequence conservation but functionally negligible?

The last experimental chapter of this thesis (**Chapter 6**) addresses a part of MscL that is underrepresented in literature. Although the carboxyl-terminus of MscL contains a motif that shows the highest level of conservation, its functional role remains enigmatic. Only a handful of studies have addressed this part of the protein and the level of information is rather sparse, due to discrepancies. While some studies propose this part of MscL to be a stable associated bundle of the five subunits throughout the course of gating (86, 142), others have reported it to dissociate (147). Here, we report supporting data for the stable conformation, which might have a supporting function in initializing the closing of the channel after gating.

Perspectives

This thesis has mainly dealt with biochemical and electrophysiological characterization of MscL using a new approach of heteropentameric MscL. Thereby, we have provided a new method to study homooligomeric proteins and by applying it we obtained new insights into the gating mechanism of MscL. Although our approach eliminated some of the experimental restraints, the used analysis techniques have their limitations, too. Applying state-of-the-art techniques, like EPR spectroscopy, in combination with single-subunit resolution of heteropentameric MscL would be the next step in gaining insights into the gating mechanism. This method would provide information about local changes in the protein backbone and deliver a high-resolution model for the structural rearrangements upon gating.

However, this project started with the question: “How do mechanosensitive channels sense force?” (PhD project proposal, 2007). A question that has

not been addressed within this thesis, but is of particular importance. Mechanosensitive channels are ubiquitous in nature and serve important processes such as touch and hearing, but also regulation of blood pressure (77). In these days, were cardiovascular diseases are responsible for the majority of deaths, molecular insights into the fundamental basis of blood pressure regulation might be of enormous value. MscL, which has served a simple, but suitable, model for mechanosensation throughout the years (58, 124), might play an important role in elucidating how mechanosensors 'feel' tension. So, addressing how MscL senses tension in the membrane and whether these principles are valid for other mechanogated channels provides one of the major challenges for the future.